The Fluorometric Assay of Soil Enzymes

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THE FLUOROMETRIC ASSAY OF SOIL ENZYMES[1]

INTRODUCTION

Two fluorometric assays were investigated: phosphatase and leucyl amino-peptidase (peptidase). In general, the enzymic activities were detectable after short incubation times (usually 1, but at times 4 hours) and required relatively small quantities of soil (from 10 to 100 mg soil per ml of reaction mixture).

The assays were complicated by the native fluorescence of soil, an apparent quenching of fluorescence by soil in the case of the phosphatase assay, and the association of enzymic activity with soil particles.

THE PHOSPHATASE ASSAY

The principle of the assay is based upon the hydrolysis of α-naphthol phosphate to α-naphthol and inorganic phosphate. The formation of α-naphthol is measured by determining the fluorescence at 460 mu when suitably treated reaction mixtures are activated with 336 mu light.

In practice, fluorescence was measured in a Turner model 111 Fluorometer, using a Corning 7-60 filter, peaking at 360 mμ, as the source of the exciting light, and a Wratten 47B filter, peaking at 436 mμ, in order to isolate the emitted light.

In order to screen the distribution of soil phosphatases, it was necessary to define conditions so that the observed phosphatase activity reflected the concentration of phosphatases in the soil. For these experiments, a reference soil was obtained from a cultivated plot, air dried, sieved through a coarse

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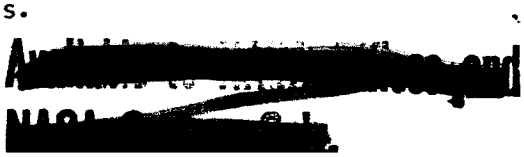
1. Soil enzymes are defined as heat labile substances catalyzing the decomposition of appropriate substrates. Thus no distinction is made between enzymic activity which arises directly from bacteria and soil enzymes, either free or attached to soil particles. Indeed, no distinction is made between biological and abiological heat labile catalysts.



screen, and stored in a closed jar at room temperature. This soil, designated as LL soil, has been used over a period of 18 months, and during that time the phosphatase activity has remained relatively constant except for a small decrease in activity which occurred during the first few months of storage. The data obtained in these experiments is contained in Appendix A. For comparative purposes, several other soils have also been included.

Based upon the data in Appendix A, the following conditions were employed in assaying soil phosphatases: 10 μmoles of α-naphthol phosphate, 200 μmoles of an appropriate buffer, and 20 (or at times 200) mg soil were incubated in a total volume of 2 ml. The reaction mixtures, incubated in 30 ml beakers, were shaken on a rotary shaker at 70 excursions per minute at room temperature, which varied from 24 to 28° C. At the end of the desired incubation period (usually 1 hour), the reaction mixtures were centrifuged for 10 minutes at 4800 RPM (approximately 3000 x g) on a Servall Table Model Centrifuge, using the SPX Rotor. Appropriate aliquots of the supernatant fluid were diluted to 4 ml. with distilled water and sufficient 1 N sodium hydroxide was added so as to insure that the resulting pH would be 10 or greater (usually 0.2 ml). Controls, consisting of substrate in buffer, and soil in buffer, were included with every determination, and the data represents net activity corrected for fluorescence arising in the controls.

A total of 59 soil samples were screened for phosphatase activity. They were obtained either by individuals on field trips or from the Soil Survey Laboratory of the University of California, Berkeley. Since, in so far as is known, no particular precautions were taken to collect these samples aseptically, and the history of these samples is not known after collection, an uncertainty exists as to how much of the observed phosphatase activity is endogenous and how much represents exogenous activity. The sources of these soils is listed in Appendix B.

A summary of the net phosphatase activity of the soil samples is presented in Appendix C. There is no correlation between the pH of the soil (as determined in a 1% suspension of soil in distilled water) and the pH at which the greater phosphatase activity occurs. 86% of the soils exhibited phosphatase activity when assayed at pH 7.6; 95% of the soils exhibited phosphatase activity when assayed at pH 5.6. 3% failed to exhibit phosphatase activity in either buffer, but did so when assayed in distilled water. In 75% of the cases, phosphatase activity was less when the assay was carried out in distilled water as compared to when the assay was carried out in a buffer. That this may involve more than a pH effect is suggested in that many cases the pH of soil-water suspensions was not too far removed from the pH of the buffered reaction mixtures.

In summary, there appear to be two kinds of phosphatase activity in soil: one which exhibits maximum activity in an alkaline environment and one which is more active in an acidic environment. The pH of the soil does not guaranty which of the two kinds of phosphatase activity will be present. Buffers enhance phosphatase activity in a way which suggests that their role may be more than that of stabilizing pH.

The phosphatase assays were complicated by the presence of a "blank" fluorescence associated with the soil. In order to ascertain the distribution of this soil fluorescence, a number of soil samples were examined for native fluorescence. In addition to determining the native fluorescence at the excitation and emission wavelengths characteristic of α-naphthol, the fluorescence of the soil samples was also determined at the excitation and emission wavelengths characteristic of fluorescein. The data is presented in Appendix D.

The background fluorescence of soil at the α-naphthol wavelengths is from about 1 to 2 orders of magnitude greater than the fluorescence observed at the fluorescein wavelengths. There was no direct correlation between the phosphatase activity of a soil and the background fluorescence. This is surprising in that one might predict that soils containing high phosphatase levels would contain relatively large bacterial populations, and that high levels of organic material, which would be expected to contribute to soil fluorescence, would be associated with these conditions.

Comparing the native soil fluorescence at 336 mμ activation - 460 mμ emission with the first hour of soil phosphatase activity, the background fluorescence turns out to be equivalent to about 5% of the net soil phosphatase activity. Assuming that these soils contain approximately 10^8 bacteria per gram, then the background fluorescence of a 10 mg soil sample is equivalent to the first hour activity of from 10^4 to 10^5 bacteria.

During the preliminary studies on the phosphatase assay, soils were observed to "adsorb" α-naphthol. This effect was particularly noticeable when alkaline soils were employed. While screening a number of ions as phosphatase inhibitors, several were observed to quench the fluorescence of α-naphthol. The ability of a number of substances to quench the fluorescence of α-naphthol was further investigated. The data (Appendix E) proved rather surprising. Thus mg (+2), which at concentrations less than 5×10^{-3} had no effect upon α-naphthol fluorescence, enhanced fluorescence at a concentration of 5×10^{-3} M and quenched at 5×10^{-2} M. Na (+1), at concentrations as high as 2.5 M (15 w/v) was without effect. Fe (+3), Mn (+2), and Hg (+2) quenched at 5×10^{-4} M. Co (+2) quenched fluorescence at extremely low concentrations; at 5×10^{-5} M, a 50% inhibition of fluorescence was observed.

The data with Co (+2) was complicated by the strong absorption of Co which occurred in the region of α-naphthol fluorescence. These interactions of ions with α-naphthol fluorescence were not further investigated. However, it is tempting to speculate that the quenching of α-naphthol fluorescence by soil and the buffer enhancement of phosphatase activity may be related to the ion effects.

As previously mentioned in Appendix A, the rate of phosphatase activity could be enhanced by shaking reaction mixtures. This suggested that soil phosphatases were attached to soil particles and that shaking led to more intimate contact between substrate and phosphatase.

In an attempt to further investigate this problem, the following experiment was carried out. Soil was separated into two fractions: a "soil supernatant" fraction and a "residual soil" fraction. This fractionation presumably separated the phosphatases associated with soil particles from those which were "free floating". Both fractions were assayed for phosphatase activity; the data is presented in Appendix F.

A total of five soils were examined. Essentially all the activity associated with soil could be accounted for in the residual soil fraction. The activity of the soil supernatant fraction amounted to about 10% of the total soil activity. As will be described subsequently, analogous results were obtained with the peptidase assay.

The data clearly locates the site of soil phosphatase activity as being associated with the residual soil fraction. Whether the residual soil activity represents adsorbed enzyme, activity arising from enzymes associated with bacteria per se, or a combination of both possibilities is uncertain.

The failure to detect significant levels of phosphatase activity in soil supernatant fractions suggests the following alternate possibilities:

(1) the assay may not be as sensitive as supposed since direct counts of the supernatant fraction yields bacterial populations of the order

of 10^7 per ml of reaction mixture (see Appendix F, Table 2).

(2) the organisms in the soil supernatant do not possess phosphatases and are different than those in the residual soil.

(3) only a small percentage of the free floating bacteria are living.

(4) the phosphatase activity of the residual soil represents not only attached bacteria but considerable free enzyme adsorbed to clay fractions, the accumulated enzyme history of that soil, and is high because of the accumulated high level of enzyme associated with the fraction.

Conclusions (1), (2), and (4) suggest that the assay is not a sensitive one in the sense that it can detect small bacterial populations over relatively short periods of time. Conclusion (3) clearly points out the fallacy of using direct counts in order to determine the bacterial populations in soil so as to reduce activity from change per mg of soil to change per bacterium (the previously used CML unit). Conclusion (3) also suggests that concentrating the free floating material may not concentrate the biologically interesting material.

THE PEPTIDASE ASSAY

The presence of soil aminopeptidases was assayed using L-leucyl-β-naphthylamide as the substrate. The hydrolysis of the substrate yields β-naphthylamine which fluoresces at 420 mμ and possesses two activation maxima: 310 mμ and 70 mμ. Unlike α-naphthol, β-naphthylamine fluoresced without the addition of sodium hydroxide: maximum fluorescence occurred at pH 7 with half maximum fluorescence occurring at approximately pH 4.

Preliminary experiments, using LL soil, were carried out in order to determine those conditions which would result in assays where the rate of peptidase activity reflected the enzyme content of the soil. These conditions were not too different from those employed in the phosphatase assay (Appendix G).

Thus, the maximum rate of peptidase activity occurred at pH 8 and at a substrate concentration of approximately 0.001 M. The rate was proportional to the quantity of added soil from 0 to 100 mgs of soil per ml. Thus, a typical reaction mixture used in a peptidase assay contained the following additions in a total volume of 2 ml: 2 μmoles L-leucyl-β-naphthylamide, 200 μmoles Tris buffer, pH 8, and from 20 to 200 mg of soil. The reaction mixtures were incubated and subsequently treated in the same manner as were the phosphatase reaction mixtures except that no sodium hydroxide was added to the diluted, centrifuged reaction mixtures. Appropriate controls were run, and all data represents net fluorescence after correction for fluorescence arising in the control tubes.

Thirty-five soils were surveyed for peptidase activity. These were the same ones that were employed in the phosphatase assay. A summary of the data is presented in Appendix H. Of the 35 soils examined, 3 did not exhibit peptidase activity; 2 of these inactive soils (I and S4) were low in phosphatase activity. Four soils exhibited greater peptidase activity than phosphatase activity; 3 of these soils contained about the same level of phosphatase activity as LL soil. The peptidase activity of the remaining soil samples varied from about 3% of the phosphatase activity of the soil to peptidase activity which was equal to the phosphatase activity. However, the average peptidase activity was about 20% of the phosphatase activity. The lack of correlation between phosphatase and peptidase activity was rather extreme so that it proved difficult to predict from the phosphatase activity just what the peptidase activity would be. For example, soils A and B which differed by a factor of approximately 4 with respect to their levels of phosphatase activity exhibited essentially identical levels of peptidase activity. Soils LL and N, which contained about the same levels of phosphatase activity

differed by a factor of approximately 8 in their levels of peptidase activity. This lack of correlation is not too surprising, and suggests that: the two enzymes are not necessarily associated with the same microorganisms; the nutritional differences in the soils are such so as to result in differential production of these two enzymes; the enzymes may have different substrate specificities; the substrate specificities may be the same but the enzymes may differ in turnover numbers.

As in the case of the phosphatase assay, the peptidase assay was plagued by the presence of a relatively high fluorescence associated with soil. However, unlike α-naphthol, β-naphthylamine was not "adsorbed" by soil.

The substrate exhibited about the same stability in solution as did α-naphthol phosphate. Thus, when sterile L-leucyl-β-naphthylamide (0.2 µmoles) was incubated at room temperature at pH 6, approximately 0.009 mµmole decomposed per hour (0.0045% per hour). This represented a significant background problem in the case of soils having low levels of peptidase activity. For example, in the case of soils S3, S5, and S9, the non-enzymic rate of hydrolysis was equal to about 18% of the net peptidase activity. In the case of soil E, which was the most active soil, the rate of non-enzymic hydrolysis was about 0.15% of the net activity. However, the stability data was equivocal, and these numbers should be looked at as first approximations.

Three organisms were assayed for peptidase activity. As indicated in Appendix J, peptidase activity was detected in all the organisms, although the BS-19 strain of B. subtilis was relatively low in activity. These studies were carried out in a medium which was favorable for the expression of enzyme activity. What levels could be expected in a synthetic medium containing no conceivable peptidase substrates (e.g., a mineral salts and sugar medium) is not known, but would be worthy of investigation so as to establish some basal level of enzyme activity.

In summary, the peptidase assay, although less sensitive than the phosphatase assay, would appear to be a relatively good candidate as an ancillary life detection assay, and warrants further investigation. It should be noted that activation and fluorescent filters were used in these measurements with maxima at 365 mμ and 435 mμ. This results in underestimating the peptidase activity by an amount less than 10% and does not take into account activation at 310 mμ which would give over 150% increase in sensitivity.

The problem of soil fluorescence may be amenable to solution by using fluorogenic peptidase substrates in which the fluor is activated in the visible regions of the spectrum. There are a number of dyes which meet this requirement such as acridine-type dyes.

The advantages of the peptidase assay compared to the phosphatase assay, at the present stage of development, are that: the fluor is not adsorbed by soil; the fluor fluoresces maximally at the pH of the reaction mixture, and in fact, fluoresces maximally from pH 6 to pH 10. This, coupled with the apparent pH sensitivity of the enzyme, suggests that biological nature of the reaction may be tested merely by increasing the hydrogen ion concentration. For example, peptidase activity was completely inhibited at pH 5, although the fluorescence of β-naphthylamine was reduced about 15% at that pH.

Appendix A

Factors Influencing the Rate of Phosphatase Activity

Fig. 1: The Effect of pH

The following additions were made up to a total volume of 2 ml: 10 μmoles α-naphthol phosphate; 200 μmoles of Tris acetate buffer at the desired pH; and 20 mgs of soil. The reaction mixtures were shaken at room temperature (24-28° C) for 1 hour, centrifuged, and the supernatants diluted 1:4 with distilled water. 0.2 ml 1 N NaOH was added to the diluted supernatants and α-naphthol was determined fluorimetrically.

Fig. 2: The Effect of Substrate Concentration

All conditions were identical as in Figure 1, except the substrate concentration was varied and all reaction mixtures were buffered at pH 7.6.

Fig. 3: The Effect of Soil Concentration

All conditions identical as in Figure 1 except the soil concentration was varied and the reaction mixtures buffered at pH 7.6.

Fig. 4: The Effect of Time

All conditions identical as in Figure 1 except that the reaction mixtures were buffered at pH 7.6 and allowed to incubate for the indicated time periods.

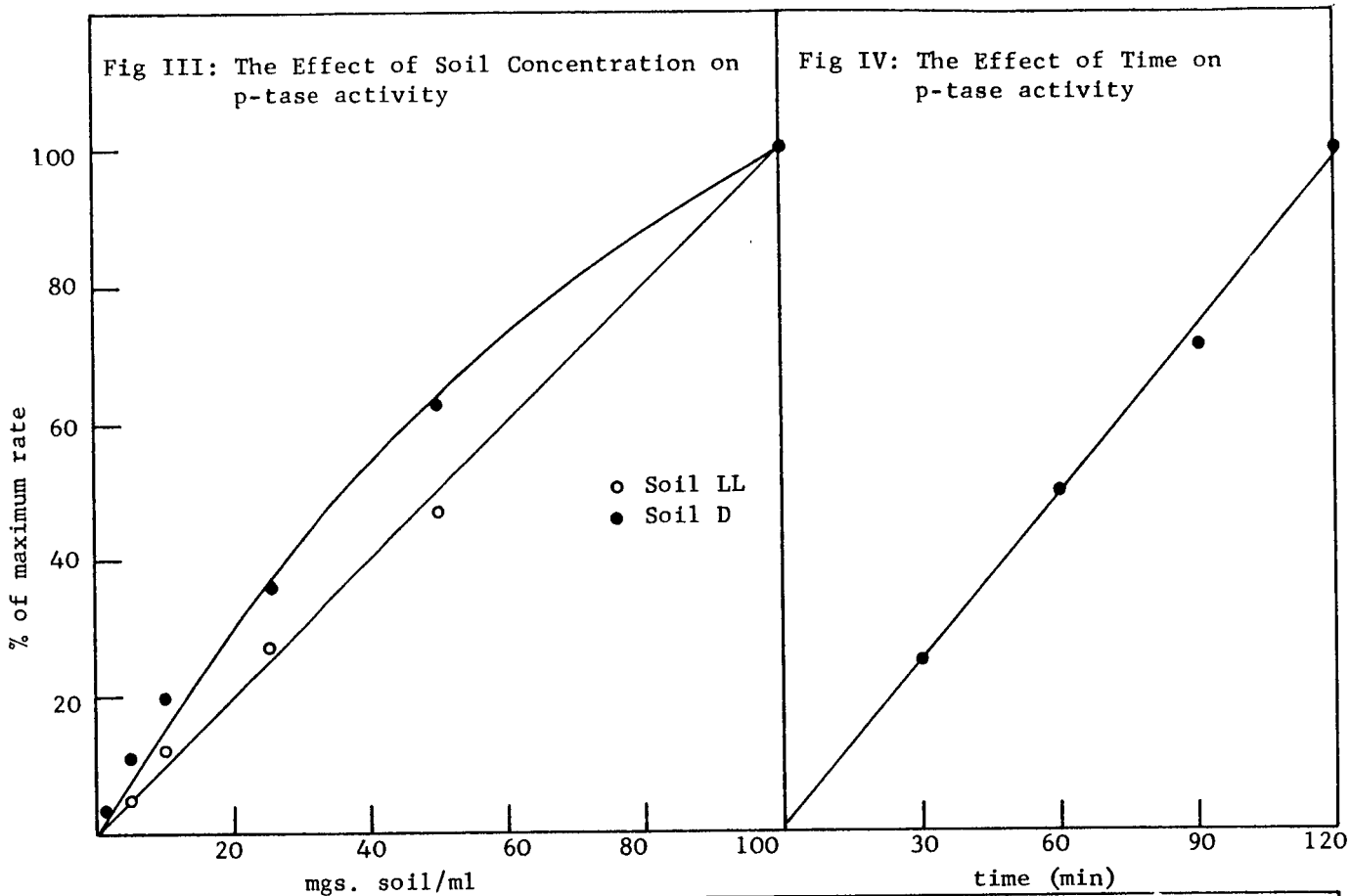


Fig III: The Effect of Soil Concentration on p-tase activity
% of maximum rate
100
80
60
40
20
Soil LL
Soil D
20
40
60
80
100
mgs. soil/ml

Fig IV: The Effect of Time on p-tase activity
30
60
90
120
time (min)

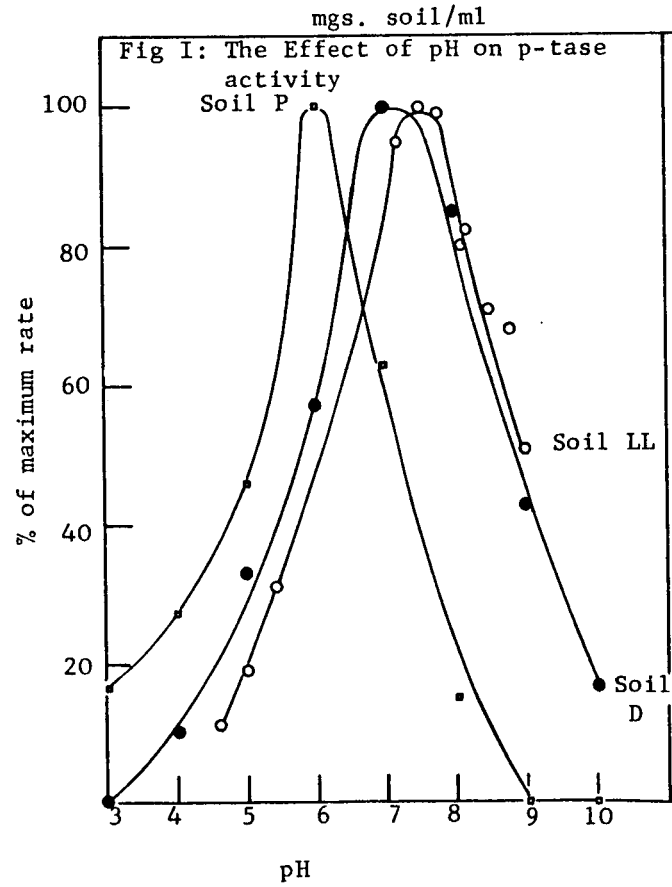


Fig I: The Effect of pH on p-tase activity
% of maximum rate
100
80
60
40
20
Soil P
Soil LL
Soil D
3
4
5
6
7
8
9
10
pH

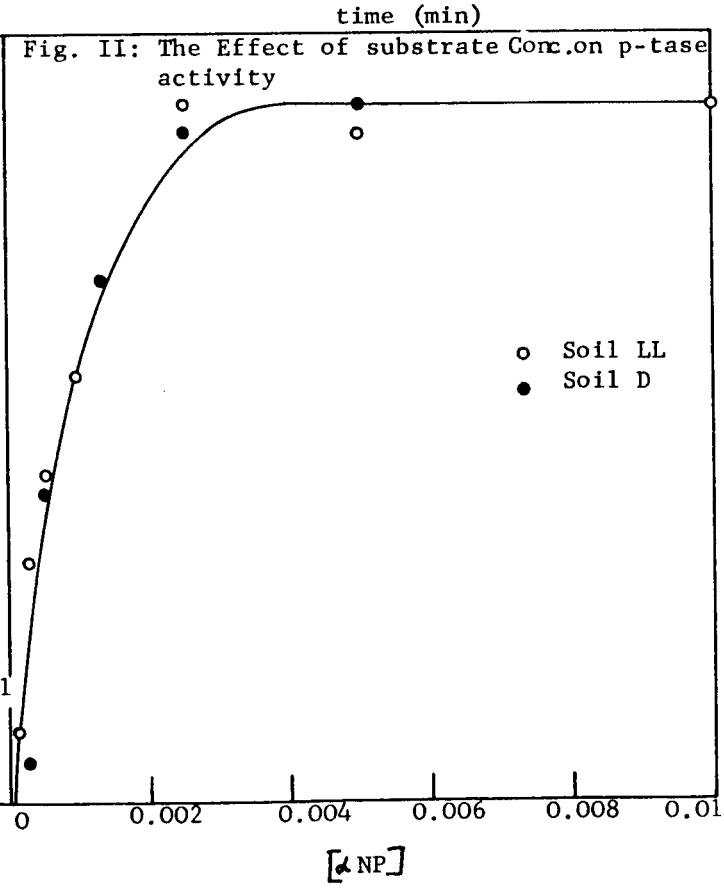


Fig. II: The Effect of substrate Conc.on p-tase activity
Soil LL
Soil D
0
0.002
0.004
0.006
0.008
0.01
[NP]

TABLE I

The Effect of Temperature Upon Rate of Soil Phosphatase Activity

Conditions	α-N (mμM/ml/lu) Temperature (C)				
	4	9	28	38	50
Complete	5.3	5.3	7.7	10.3	18.1
- sub	0.7	0.7	0.8	0.9	1.1
- soil	3.2	3.4	3.4	3.9	4.6
Δ (in mμM/ml)	+1.4	+1.2	+3.7	+5.5	+12.4

Conditions: The following additions were incubated in a total volume of 2 ml: α-naphtholphosphate 10 μmoles; Tris HCl, pH 7.6, 200 μM, soil #LL 20 mg. The reaction mixtures were incubated for 1 hour at the indicated temperatures in screwcap tubes without shaking.

TABLE II

The Effect of Temperature Upon Soil Phosphatase Activity - Survey of Soils

Soil	Δ α-N (mμM/ml/lu) Temperature (C)	
	24	50
A	+ 7.6	+23.4
B	+ 3.1	+ 6.9
L	0	+ 3.7
S_8	+ 1.7	+ 2.3
T_1	+ 1.4	+ 5.9

Conditions: The following additions were incubated in a total volume of 2 ml: α-naphtholphosphate 10 μmoles; Tris HCl, pH 7.6, 200 μmoles, soil, 20 mg. The reaction mixtures were incubated at room temperature in screwcap tubes without shaking. Incubation time: soils A, B, L and T_1, 1 hour; soil S_8, 4 hours. The data represents net change after correction for fluorescence arising in the control tubes.

TABLE III

The Effect of Shaking Upon Rate of Soil Phosphatase Activity

Expt. No.	$+\Delta \alpha$ N (mμM/Ml) Conditions	
	Sessile	Shaking
1	2.0	3.0
2	2.0	3.1
3	2.7	3.0
Average	+2.2	+3.0

Conditions: The following additions were incubated in a total volume of 2 ml: α-naphtholphosphate 10 μmoles; Tris HCl, pH 7.6, 200 μmoles; soil #LL 20 mg. Incubation time was 1 hour at room temperature. The data represents net change after correction for fluorescence arising in the control tubes.

TABLE IV

The Effect of Sterilization of Reagents Upon the Stability of α-Naphtholphosphate

Expt. No.	mμM α-naphthol in α-naphtholphosphate		
	Zero Time	After 1 hour	
		Sterile	Contaminated
1	6.3	6.1	6.4
2	6.4	6.5	6.8
3	6.3	6.3	7.0
Average	6.3	6.3	6.7

Conditions: The following additions were incubated in total volume of 2 ml: α-naphtholphosphate 10 μmoles, Tris. HCl, pH 7.6, 200 μmoles. The reaction mixtures were incubated for 1 hour at room temperature without shaking. α-naphtholphosphate was sterilized by filtration through a Morton bacterial filter. The sterile samples were incubated in sterilized screwcap tubes; the contaminated samples were incubated in screwcap tubes which had not been sterilized.

Appendix B

KEY TO SOIL SAMPLES OBTAINED FROM SOIL SURVEY LABS, UNIVERSITY OF CALIFORNIA

(Key prepared by E. Packer)

Soil Series of California Formation and Characteristics
Key for Identification - Pedological Classification

Code No.	County	Year	Depth	Type
T1	Glenn	'58	0-9"	
T2	Glenn	'58	0-8"	
T3	Glenn	'58	0-5"	
T4	Glenn	'58	0-9"	
T5	Tehama	'58	0-1"	
T6	Tehama	'58	0-1"	
T7	Tehama	'58	0-1"	
T8	Tehama	'58	0-7"	
T9	Tehama	'58	0-1"	
T10	Tehama	'58	0-3"	
T11				
T12	Tehama	'58	0-5"	
T13	Tehama	'58	0-1.5"	
T14	Lake	'54	0-8"	
T15	Lake	'54	0-4"	
T16	Lake	'54	0-4"	
T17	Lake	'54	0-6"	
T18	Lake	'54	0-4"	
T19	Lake	'54	0-3"	
T20	Lake	'54	0-7"	
T21	Lake	'54	0-5"	
T22	Lake	'54	0-3"	
T23	Lake	'54	0-5"	
T24	Shasta	'61	0-3"	
T25	Shasta	'60	0-1"	
T26	Shasta	'61	0-5"	
T27	Shasta	'61	0-2"	

KEY TO SOIL SAMPLES (continued)

Code No.	County	Year	Depth	Type
T28	Humboldt	'60	0-1.5"	
T29	Humboldt	'60	0-10"	
T30	Fresno-Sierra	'59	0-2"	
T31	Fresno-Sierra	'59	0-1"	
T32	E. Fresno	'58	0-3/4"	
T33	E. Fresno	'58	0-4"	
T34	E. Fresno	'58	0-4"	
T35	E. Fresno	'58	0-2"	
T36	E. Fresno	'58	0-5"	
T37	E. Fresno	'58	0-5"	
T38	E. Fresno	'59	0-3"	
T39	Fresno	---	---	
T40	Humboldt	'55	0-8"	
T41	Glenn	'56		
T42	Tehama	'56	0-2"	
T43	Alameda			
T44				

Soils Data

II A. Collected 5/13/62. Grey hard pan from hillside near beach at San Gregorio. California. This soil is hard and compact where sampled. Rather claylike in texture, supported no vegetation. Old code 12 - 5/13

II B. Collected 5/13/62. Brownish loam from artichoke field under cultivation. Collected under end around plants. This soil appeared to be well drained. Field lies across highway 1 from beach at San Gregorio. Old code 9 - 5/13

II C. Collected 5/13/62. This soil of a hard grey black description came from a field by the side of the road to the beach from the town of San Gregorio. It supported a heavy cover of foxtails and other grasses. Old code 8 - 5/13.

II D. Collected 5/13/62. Soils taken beneath conifer by roadside on road to Skyline (La Honda Road). This soil should have a low pH. Taken from about 2/3 of the way up to Skyline Blvd. Old code 5 - 5/13

II E. Collected 5/13/62. This soil taken from creek bed at mouth of rusted drain pipe on Stanford campus, 1 block from Mayfield on Frenchmen's road across from Frank Lloyd Wright house. This soil has a very high Fe_2O_3 content and appears reddish brown. Where collected was saturated with water. Old code 1 - 5/13.

II F. Collected 5/13/62. This soil, a yellowish-orange clay was collected from a road side bank on the ascent to Skyline Blvd. (Highway 84 from Palo Alto). Old code 4 - 5/13

II G. Collected 5/13/62. This soil was a whitish hard pan collected from a hillside near beach at San Gregorio. Very similar to II A, but of a lighter color. Old code 11 - 5/13

II H. Collected 5/13/62. On Frenchmen's Road. 1 block from Mayfield on Stanfore campus. Across road from II E. This soil is a weathered, decomposed Ozonite, a claylike sand, and showed no evidence of supporting any vegetation. Old code 2 - 5/13.

II I. Collected 5/13/62. This is sand from the non supratidal or high intertidal zone of the beach near San Gregorio, Calif. Old code 10 - 5/13

II J. Collected 5/20/62. This sample comes from a vineyard under cultivation in San Martin, Calif. May have Cu^{++} from sprays. A grayish brown soil. Old code 3 - 5/20

II K. Collected 5/20/62. Taken from raspberry orchard under irregation in San Martin. Soil type similar to II J. Old code 4 - 5/20

II L. Collected 5/20/62. This material is the mud in contact with the brine of an evaporation basin of the Leslie Salt Co., Harbor Road, Redwood City. Calif. Brine, also collected near site was pink and contained brine shrimp. Old code 1 - 5/20

II M. Collected 5/13/62. Taken at historical marker 478, Old Saw Mill (LaHonda Road) from a bank by roadside, under Eucalyptus tree. Therefore, soils should be charged with the essential oils of the Eucalyptus. Old code 3 - 5/13.

II N. Collected 5/20/62. This soil consists of dirt and tailings from mercury mine in this area. However, this was not taken from tailing piles directly, but from a pile by a house. However, there should be some Hy in soil. Taken from Tobar property. Old code 5 - 5/20. New Almaden, Calif.

II O. Collected 5/13/62. Bank on roadside, after Woodhaven Camp turnoff on Highway 84 from Skyline toward La Honda. This sample is a weathered, exposed, decomposed granite. Across road from Bulocco residence. Old code 7 - 5/13

II P. Collected 5/13/62. Taken from roadside bank just past Skyline and before Woodhaven on Highway 84. Another decomposed weathered granite soil. Old code 6 - 5/13.

II Q. Collected 5/20/62. Algel scum from impoundment neighboring evaporation basin of II L. Water was clear, no evidence of shrimp or reddish color. May be partly rain water rather than brine. Old code 2 - 5/20.

LL - Lane Library air dried collected in 1961 by Packer

FA - Fresno Black alkali soil collected in the 1930's by C.F. Shaw

MDC - Mohave Desert soil from China Lake obtained 7/27/62 from Soffen (JPL).

S_{13} - Sand from Thermal, California ("ancient sea sand") obtained from Soffen (JPL) 7/19/62

GM1 - AC fine (Lundgren, General Mills, see letter 7/24/62)

GM2 - AC course (Lundgren, General Mills, see letter 7/24/62)

GM3 - NBS Fly Ash (Lundgren, General Mills, see letter 7/24/62)

Sand Samples from Dr. Lederberg

S-1 Owens Valley

S-2 Panament Dry Lake

S-3 Devils' Golf Course - salt pan

S-4 Salt pan near Golden Canyon. No vegetation 7500 feet, Death Valley

S-5 Rubble near F.C. 1 m - salt marsh

S-8 Death Valley - Meadow near Dante View

S-9 Death Valley - Dunes roots

S-10 Death Valley - Dunes surface

S-11 Death Valley - Dunes - 1-3 inches

S-12 Death Valley - Dunes - 6-12 inches

Appendix C

TABLE V

The Distribution of Phosphatase in Soils

Soil	pH	+ΔαN (mμM/hr./10 mgs. soil)		
		in T. HCl	in NaAc/HAc	in H_2O
LL	7.1	3.0	1.0	0.3
A	6.9	11.7	4.2	5.9
B	7.2	2.8	0.4	-
C	7.3	63.8	10.6	3.4
D	6.9	9.2	2.9	2.5
E	8.5	3.8	2.0	3.0
F	6.3	3.0	6.1	2.2
G	7.1	6.4	0.2	-
H	7.8	-	3.7	1.1
I	7.8	0.5	1.4	-
J	7.1	16.5	4.6	2.7
K	7.1	6.5	1.4	0.7
L	7.4	0.9	1.2	1.0
M	7.3	17.4	5.3	3.6
N	7.7	3.9	2.1	2.8
O	7.3	5.9	1.2	1.6
P	5.3	0.5	4.0	-
S_1	8.2	0.1	-	0.3
S_3	9.0	-	-	0.3
S_4	8.8	-	-	0.5
S_5	8.8	-	0.9	0.5
S_8	8.4	0.4	0.3	0.3
S_9	8.6	-	0.3	0.6
S_{10}	8.3	-	0.3	0.4
S_{11}	8.3	-	0.1	0.1
S_{12}	8.3	-	2.0	0.6
S_{13}	7.1	0.02	0.03	0.05

TABLE V (Continued)

Soil	pH	+ΔαN (mμM/hr./10 mgs. soil)		
		in T. HCl	in NaAc/HAc	in H_2O
T_1	7.3	3.9	0.7	0.1
T_2	7.1	4.7	1.1	1.0
T_3	6.9	14.5	2.5	1.7
T_4	8.6	1.2	1.2	1.4
T_5	6.6	16.0	14.2	9.2
T_6	6.8	13.3	5.4	3.0
T_7	7.1	11.4	3.1	2.9
T_8	6.6	20.3	8.9	3.2
T_9	6.7	13.3	6.1	4.8
T_{10}	6.6	21.2	7.6	4.0
T_{11}	6.1	10.7	7.0	4.5
T_{12}	6.3	35.1	9.6	6.5
T_{13}	6.4	7.2	0.3	-
T_{14}	6.2	2.9	4.5	0.7
T_{15}	6.2	15.2	3.4	3.1
T_{16}	6.5	11.6	3.9	3.1
T_{17}	6.2	3.3	5.4	2.2
T_{18}	6.4	21.0	6.5	4.2
T_{19}	6.5	6.2	1.8	1.7
T_{20}	6.5	19.2	3.4	2.3
T_{21}	6.5	19.2	3.4	2.3
T_{22}	6.5	7.8	7.2	4.8
T_{23}	6.5	8.7	4.9	3.8
T_{24}	6.5	6.2	5.7	1.7
T_{25}	6.3	14.0	2.7	1.0
T_{26}	6.3	0.8	1.7	0.6
T_{27}	6.5	11.2	2.8	2.6
T_{28}	6.4	10.0	9.9	6.3
T_{29}	6.3	2.8	2.9	1.6
T_{30}	6.2	23.3	16.9	12.3
T_{31}	6.2	8.0	14.2	7.2
MDC	-	0.6	0.2	0.3

Appendix D

The Native Fluorescence of Soils

A 1% suspension (w/v) of soil was made in 0.2 M Tris HCl buffer (pH 7.6) and the suspension was shaken for 1 hour at room temperature. The soil suspensions were centrifuged and the undiluted supernatants were examined for fluorescence in an Aminco-Bowman Spectrofluormeter.

The native fluorescence of soil was first determined using 500 mμ and 520 mμ as the excitation and emission wavelengths respectively (corresponding to fluorescein fluorescence). Sodium hydroxide was then added and the fluorescence at the excitation and emission wavelengths characteristic of α-naphthol were determined (336 mμ and 460 mμ respectively). The Aminco-Bowman was standardized using known concentrations of fluorescein and α-naphthol and the native soil fluorescence was converted to concentration (fluorescein and α-naphthol equilvalents) by relating the relative fluorescence of the soil supernatants to the relative fluorescence of the appropriate standards.

Appendix D

Native Fluorescence of Soil

Soil	MμM ≈ Fluorescein	MμM ≈ ∝-Naphthol	Soil	MμM ≈ Fluorescein	MμM ≈ ∝-Naphthol
LL	0.04	0.40	T5	0.010	0.27
A	0.008	0.59	T6	0.010	0.16
B	0.034	0.57	T7	0.068	0.27
C	0.003	0.36	T8	0.010	0.20
D	0.001	0.21	T9	0.005	0.14
E	0.001	0.23	T10	0.006	0.12
F	0.004	0.09	T12	0.013	0.27
G	0.001	0.04	T13	0.033	0.15
I	<0.001	0.02	T14	0.010	0.11
J	0.006	0.23	T15	0.006	0.24
K	0.008	0.16	T16	0.005	0.17
L	0.001	0.25	T17	0.015	0.06
M	0.002	0.12	T18	0.011	0.17
N	0.001	0.26	T19	0.040	0.12
S1	0.003	0.45	T20	0.005	0.08
S3	<0.001	0.06	T21	0.013	0.14
S4	<0.001	0.08	T22	0.006	0.11
S5	<0.001	0.04	T23	0.006	0.22
S8	<0.001	0.06	T24	0.004	0.15
S9	<0.001	0.09	T25	0.005	0.16
S10	0.001	0.03	T26	0.012	0.10
S11	<0.001	0.12	T27	0.085	0.16
S12	<0.001	0.04	T28	0.022	0.23
S13	<0.001	0.03	T29	0.399	0.23
T1	0.006	0.38	T30	0.030	0.32
T2	0.033	0.14	T31	0.010	0.32
T3	0.027	0.13			
T4	0.010	0.14			

Appendix E

Effect of Ions on the Fluorescence of α-Naphthol

The following additions were made in a total volume of 4 ml: 12 mμmoles α-naphthol, 200 μmoles Tris HCl, pH 7.6, and the indicated ion so as to obtain the desired molar concentration. After 10 minutes, 0.3 ml 1 N sodium hydroxide was added and the fluorescence of α-naphthol was determined in a Turner Fluorometer.

Appendix E

Effect of Ions on ∝-Naphthol Fluorescence

Addition	Concentration (M)					
	0	5×10^{-6}	5×10^{-5}	5×10^{-4}	5×10^{-3}	5×10^{-2}
$MgCl_2$	3.1	3.0	3.0	3.0	4.2	1.2
$FeNH_4(SO_4)_2$	3.0	3.1	2.8	1.2	0	0
$MnSO_4$	3.1	3.1	2.7	1.2	0.3	0.1
$COSO_4$	3.1	2.8	1.6	0	0	0
$CuSO_4$	2.9	2.9	2.9	2.7	1.9	0
$HgCl_2$	2.9	2.9	2.8	1.8	0	0
$UO_2(Ac)_2$	3.1	3.2	3.1	1.5	0	0
$Al(NO_3)_3$	3.1	3.0	3.0	3.0	2.4	0
H_3BO_3	3.0	3.0	3.0	3.0	2.9	1.1
$CdCl_2$	3.1	3.1	3.0	3.5	0	0
NH_4Cl	3.1	3.0	3.0	3.0	3.0	1.7
Na_2MoO_4	3.1	3.1	3.1	3.1	3.0	2.8
$CaCl_2$	3.1	3.1	3.1	3.1	3.1	3.0
$ZnSO_4$	3.2	3.1	3.1	3.1	3.4	0
$Na_2HA_sO_4$	3.1	3.1	3.1	3.1	3.1	3.1
Na_3 citrate	3.1	3.1	3.1	3.1	3.1	3.1
KH_2PO_9	3.2	3.2	3.2	3.2	3.2	3.2
$BeCl_2$	2.8	3.1	3.0	2.9	2.8	0
	0	0.125M	0.25M	0.5M	1.25M	2.5M
NaCl	3.0	3.0	3.0	3.0	2.9	3.0

Appendix F

The Fractionation of Soil Phosphatase Activity

Soils were separated into two fractions: the "soil supernatant fraction" (in reality a soil suspension) and a "residual soil fraction" (that fraction of soil which settled out in 10 minutes).

The following procedure was employed to isolate the fractions: soil was suspended in 0.2 M buffer (Tris HCl, pH 7.6, in all cases except soil P which was suspended in acetate buffer, pH 5.6) at two times the desired final concentration to be used in the reaction mixtures. The soil-buffer mixture was shaken on a rotary shaker for 1 hour at room temperature. The soil-buffer mixture was then poured into conical centrifuge tubes and allowed to stand for 10 minutes. After 10 minutes, the coarse particles had settled out leaving a turbid suspension (designated as the soil supernatant) which contained fine soil particles and bacteria, many of which were motile, and hence judged viable. The soil supernatant was decanted and the residual soil was brought up to volume so that the final concentration of residual soil was two times the desired final concentration to be used in the reaction mixtures. One ml of each fraction was used as the source of phosphatase.

Table I contains the data obtained in these experiments. Table II contains direct counts of the soil supernatant fraction.

Appendix F

Table I

The Fractionation of Soil Phosphatase Activity

Soil	Conditions	Fraction		
		Soil Supernatant	Residual Soil	Untreated Soil
LL	Complete	6.0	8.9	9.0
	substrate	4.9	4.9	4.9
	soil fraction	0.9	0.9	0.9
	Δ	+0.2	+3.1	+3.2
A	Complete	5.7	14.3	12.4
	substrate	2.9	2.9	3.1
	soil fraction	1.9	1.4	1.7
	Δ	+0.9	+10.0	+7.6
B	Complete	6.8	9.2	10.7
	substrate	5.7	5.8	5.6
	soil fraction	1.3	0.7	1.1
	Δ	0	+2.7	+3.0
E	Complete	6.9	9.7	10.2
	substrate	5.5	5.6	5.4
	soil fraction	1.0	0.5	0.9
	Δ	+0.4	+3.6	+3.9
P	Complete	6.5	7.6	8.5
	substrate	5.7	5.7	5.4
	soil fraction	0.6	0.3	0.5
	Δ	+0.2	+1.6	+2.6

Appendix F

Table II

Direct Counts of Soil Supernatant Fractions

Soil	Bact/10 mg Soil
LL	3×10^7
A	7×10^7
B	5×10^7
E	2×10^6
P	9×10^6

Appendix G

Factors Influencing Peptidase Activity

Fig. 1: Effect of pH on Peptidase Activity

The following additions were made in a total volume of 2 ml: 2 μmoles L-leucyl-β-naphthylamide; 200 μmoles Tris acetate buffer at the indicated pH; 200 mgs LL soil. All subsequent conditions identical to the phosphatase assay except that no NaOH was added to the diluted centrifuged reaction mixtures. The data represents net change after correction for fluorescence arising in the controls.

Fig. 2: Effect of Substrate Concentration

The conditions were identical as those in Fig. 1 except that the reaction mixtures were buffered at pH 8, and the substrate concentration was varied. The peak at 0.001 M has not been investigated.

Fig. 3: The Effect of Soil

The conditions were identical to those used in Fig. 1 except that the reaction mixtures were buffered at pH 8, and the soil concentration was varied.

Fig. 4: Fluorometric Titration of β-Naphthylamine

0.5 mμmole β-naphthylamine was dissolved in 0.1 M Tris acetate buffer at the indicated pH. The fluorescence was determined in a Model III Turner Fluorometer, using a 7-60 excitation filter and a 47 B filter for isolating the emitted light.

Fig 1: Effect of pH on Peptidase activity

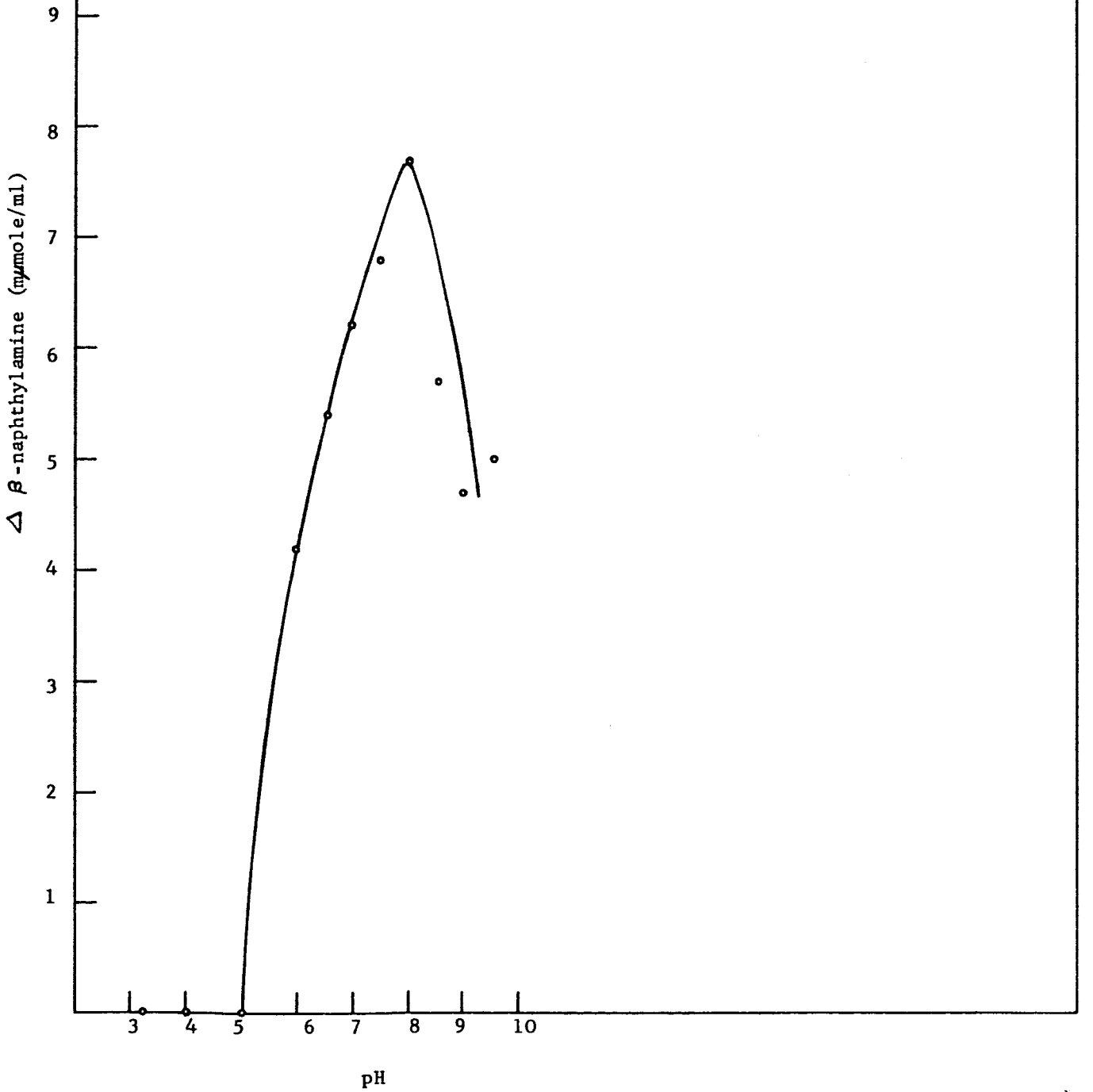


Fig 2: Effect of Substrate Concentration

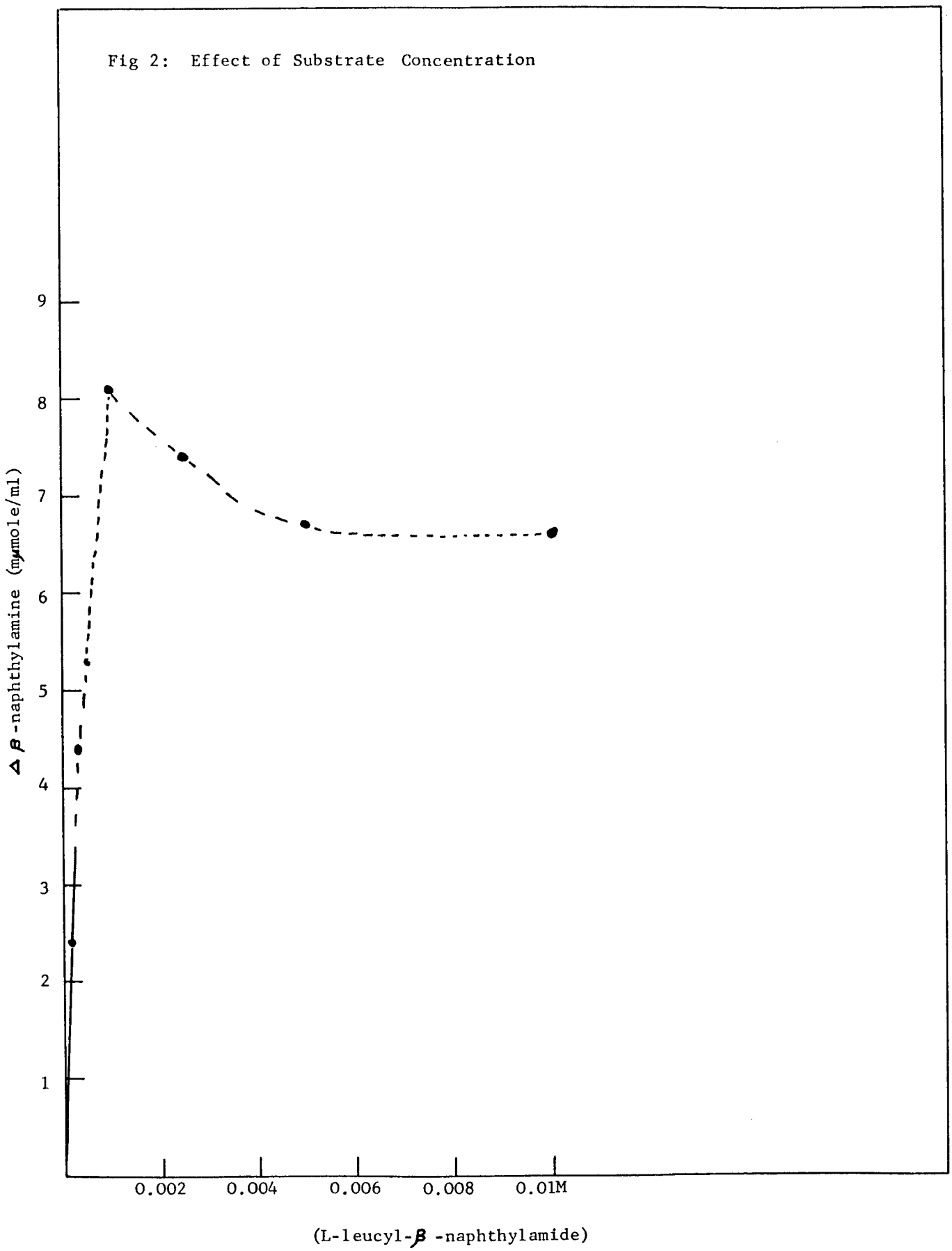


Fig.4: Fluorometric Titration of β-naphthylamine

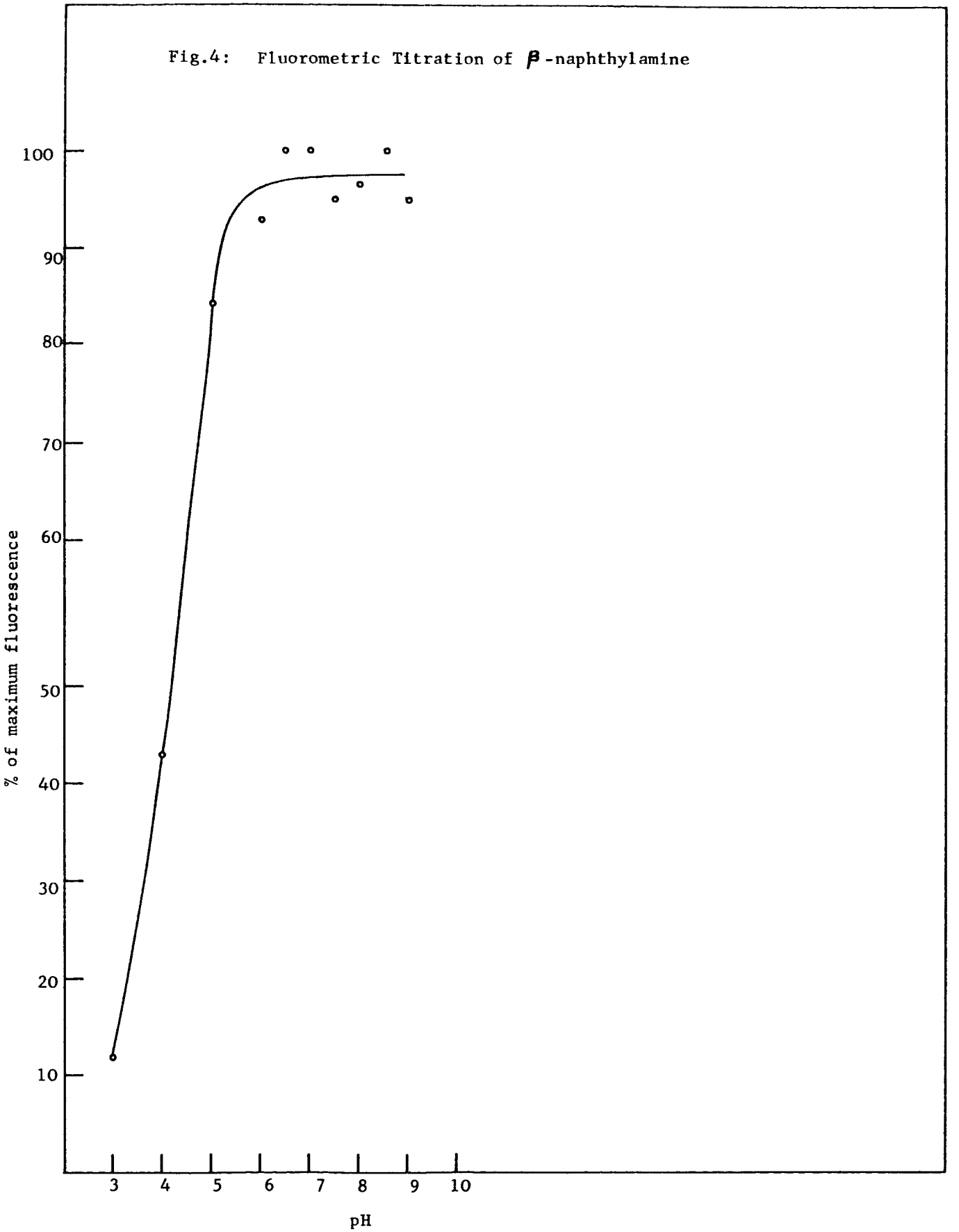
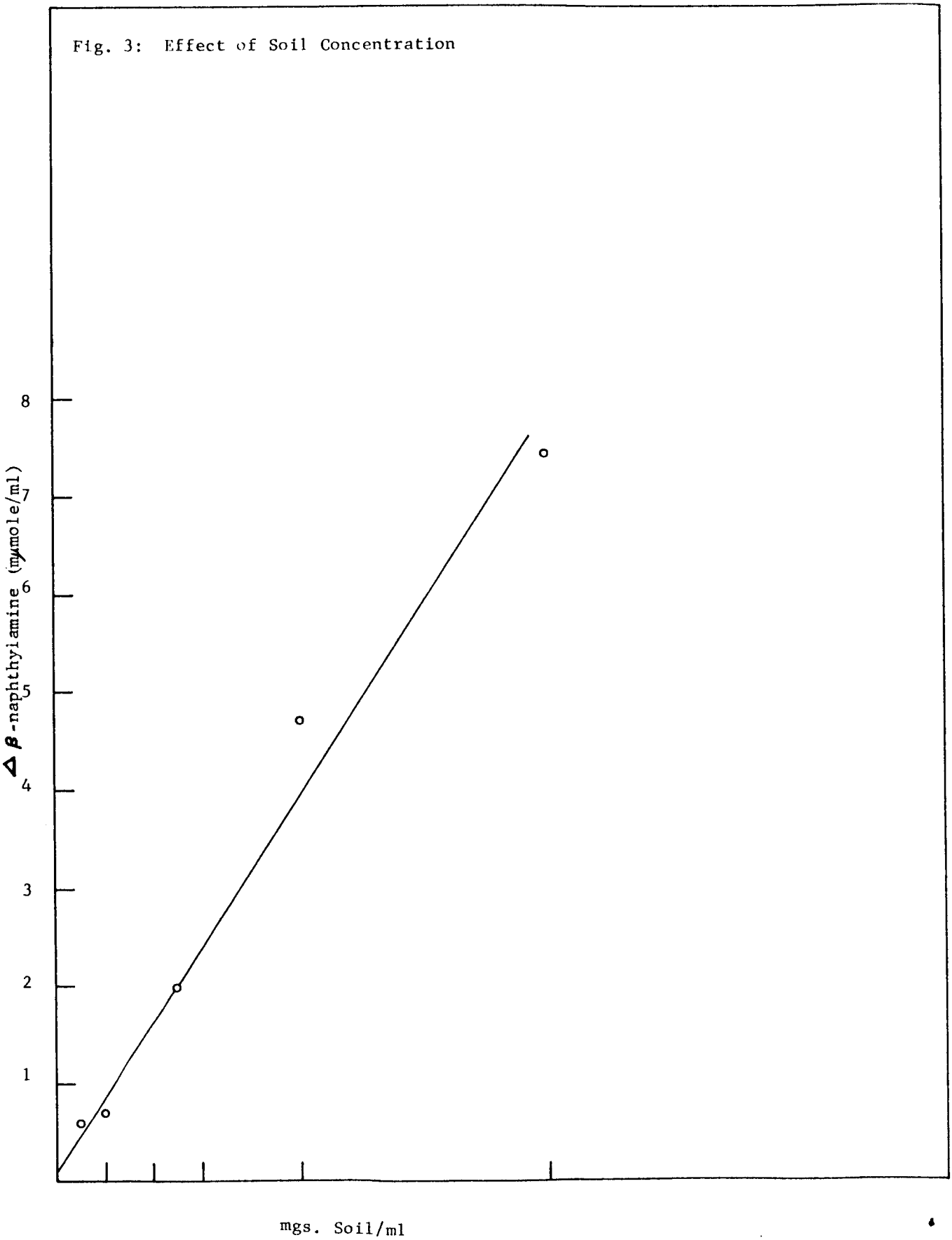


Fig. 3: Effect of Soil Concentration



Appendix J

Peptidase Activity of Pure Cultures

Organism	+α-Naphthol (in mμmoles)	+β-Naphthylamine (in mμmoles)
B. subtilis, BS19	2.5	0.8
A. fecalis	71	93
S. lutea	0	78

The organisms were grown in trypticase broth for 24 hours. The cells were harvested by centrifugation, washed three times with 0.85% saline and resuspended in 0.85% saline to yield a bacterial population of 2.5×10^7 cells per ml of reaction mixture.

The assays were carried out in standard reaction condition as previously described. The changes represent net changes, corrected for fluorescence arising in controls.

Appendix I

Localization of Peptidase Activity

Additions	Soil Fraction		
	Soil Supernatant	Soil Residue	Untreated Soil
Soil LL			
complete	2.3	3.4	4.8
substrate	1.5	0.7	1.4
soil fraction	0.3	0.3	0.3
Δ MμM/10 mg/hr	+0.3	+1.2	+1.5
Soil A			
complete	4.0	3.5	5.8
substrate	3.4	1.5	3.3
soil fraction	0.3	0.3	0.4
Δ	+0.3	+1.7	+2.1
Soil B			
complete	5.1	7.8	11.1
substrate	3.9	1.9	4.1
soil fraction	0.4	0.4	0.4
Δ	+0.8	+5.5	+6.6
Soil C			
complete	3.8	15.3	17.6
substrate	1.7	1.5	1.9
soil fraction	0.6	0.6	0.6
Δ	+0.8	+6.6	+7.4

Soil	+ β mμmoles β-naphthylamine / 10 mg soil	Phosphatase Activity[a] / Peptidase Activity
T3	2.1	6.9
T4	4.1	0.3
T5	3.9	4.1
T6	2.6	5.1
T7	5.4	2.1
T8	2.5	8.1
T9	3.4	3.9
T10	3.9	5.4

a. Phosphatase activity determined at pH 7.6

b. Phosphatase activity determined at pH 5.6

c. Phosphatase activity determined in distilled water

Appendix H

The Distribution of Peptidase Activity in Soil

Soil	+ Δ mμmoles β-naphthylamine / 10 mg soil	Phosphatase Activity[a] / Peptidase Activity
LL	0.7	4.3
A	2.0	5.9
B	1.8	1.6
C	4.9	13.1
D	1.4	6.6
E	6.1	0.6
F	0	-
G	0.2	32
H	2.3	1.6[b]
I	0	-
J	3.8	4.3
K	1.1	5.9
L	0.5	1.8
M	2.4	7.3
N	5.8	0.7
O	3.4	1.7
P	0.2	2.5
S1	0.3	1.0[c]
S2	0.5	-
S3	0.05	6[c]
S4	0	-
S5	0.05	18[b]
S8	1.5	0.3
S9	0.05	12[c]
S13	0.2	10
T1	1.2	3.3
T2	1.6	2.9